European Journal of Medicinal Chemistry 46 (2011) 4915–4923

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Overcoming human P-glycoprotein-dependent multidrug resistance with novel dihydro-β-agarofuran sesquiterpenes

Nayra R. Perestelo [a,1], María P. Sánchez-Cañete [b,1], Francisco Gamarro [b], Ignacio A. Jiménez [a], Santiago Castanys [b,**], Isabel L. Bazzocchi [a,*]

[a] Instituto Universitario de Bio-Orgánica "Antonio González", Departamento de Química Orgánica, Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez 2, 38206 La Laguna, Tenerife, Spain

[b] Instituto de Parasitología y Biomedicina López-Neyra, Consejo Superior de Investigaciones Científicas (IPBLN-CSIC), Parque Tecnológico de Ciencias de la Salud, Avenida del Conocimiento s/n, 18100 Armilla, Granada, Spain

ARTICLE INFO

Article history:
Received 24 May 2011
Received in revised form
25 July 2011
Accepted 26 July 2011
Available online 4 August 2011

Keywords:
Maytenus jelskii
Celastraceae
Dihydro-β-agarofuran sesquiterpenes
Human MDR1/P-glycoprotein inhibitors
Structure–activity relationship

ABSTRACT

Sixteen (**1**–**16**) dihydro-β-agarofuran sesquiterpenes were isolated from the fruits of *Maytenus jelskii* and evaluated against mammalian cells with a multidrug resistance phenotype mediated by the overexpression of the human P-glycoprotein. Their stereostructures have been elucidated on the basis of spectroscopic analysis, including 1D and 2D NMR techniques, CD studies, chemical correlations and biogenetic means. Eight compounds from this series were discovered as potent chemosensitizers (**1**, **2**, **4**, **6**, **8**, **9**, **11** and **14**), showing similar effectiveness to or higher than the classical P-glycoprotein reversal agent verapamil, a first-generation chemosensitizer, when reversing resistance to daunomycin and vinblastine. Detailed structure–activity relationships revealed that aromatic substituents at the 6 and 9-position of the sesquiterpene scaffold were able to modulate the intensity of inhibition.

1. Introduction

Cancer is one of the most dangerous, fast propagating with quite high mortality rate diseases of present century. Following heart disease, cancer is the second leading cause of death in the developed world [1]. One of the major problems of cancer chemotherapy is intrinsic or acquired drug resistance, and searching to overcome it has been a major effort in clinical oncology [2]. A primary mechanism of multiple-drug resistance (MDR) may due to the overexpression of the ABC (ATP-binding cassette) transporter P-glycoprotein (Pgp) in the plasma membrane of resistant cells, which mediates the efflux of MDR drugs, reducing intracellular accumulation of anticancer drugs [3]. Pgp-mediated drug transport could be inhibited or altered by a wide range of agents (named as MDR reversal agents, modulators or chemosensitizers). Verapamil, a calcium channel blocker, was the first discovered Pgp inhibitor. Since then, various reversal agents were brought to light, including first-generation Pgp inhibitors, *e.g.* quinidine, and cyclosporine A, however, they lack specificity, require high doses to reverse multidrug resistance, and are associated with unacceptable toxicities. Second-generation agents as *e.g.* valspodar, elacridar, biricodar, and dexverapamil, are more potent, specific and less toxic compared to the former inhibitors, but they show interactions with other transporter proteins. Third-generation Pgp inhibitors, *e.g.* tariquidar, zosuquidar, laniquidar, and ONT-093, have high potency and specificity for Pgp and in addition have shown no clinically significant drug interactions with common chemotherapeutic agents. However, these compounds were ineffective or toxic at the doses required to attenuate Pgp function, or induced unfavorable pharmacokinetic interactions, resulting in failure in their clinical trials. Although progress in the development of ideal Pgp inhibitors has been slow, it is believed that this approach is still realistic and promising for overcoming MDR [4].

Natural products from plant and their synthetic derivatives play an important role in the development of innovative drugs, especially for the treatment of infections and cancer. Some natural products of relatively low toxicity are reported to be inhibitors of one or more ABC drug efflux pumps, such as terpenoids, coumarins and steroids, and there is a wealth of literature demonstrating the modulator effects of plant flavonoids and carotenoids. As yet, the potential use of such natural products has not been satisfactorily explored [5].

* Corresponding author. Tel.: +34 922 318594; fax: +34 922 318571.
** Corresponding author. Tel.: +34 958 181666; fax: +34 958 181632.
E-mail addresses: castanys@ipb.csic.es (S. Castanys), ilopez@ull.es (I.L. Bazzocchi).
[1] Both authors contributed equally to this work.

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.07.048

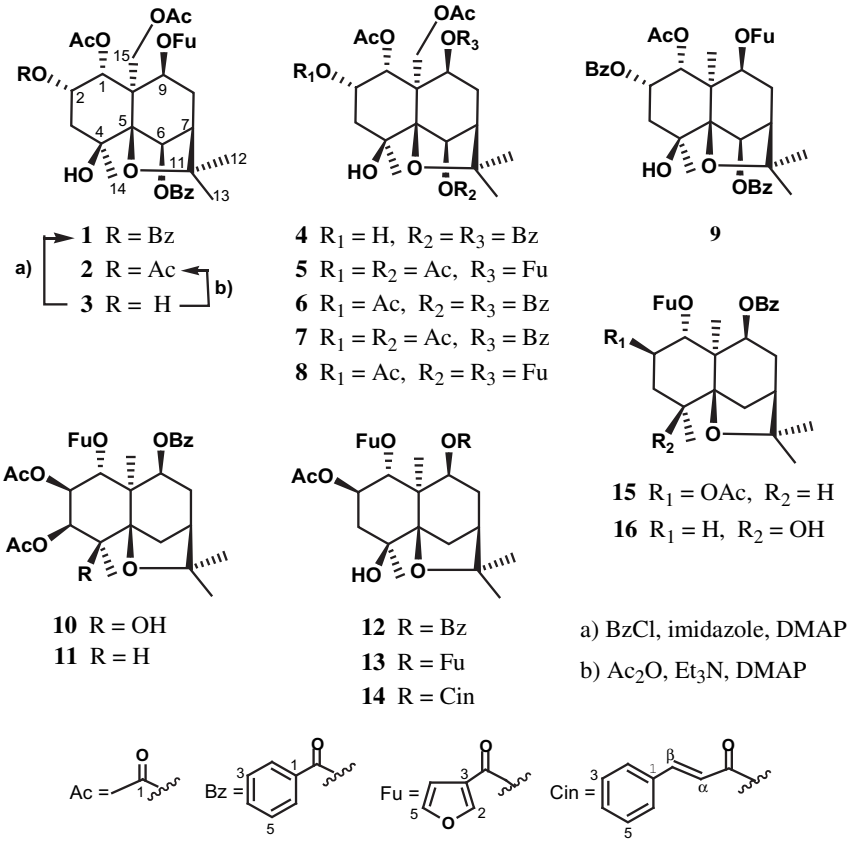
In our previous research on reversal agents of the Pgp-dependent MDR phenotype from plant, dihydro-β-agarofuran sesquiterpenes (DAS) isolated from Celastraceae species have demonstrated potent reversing effect on the cytotoxicity of the antitumoral drugs daunomycin (DNM) and vinblastin (VBT) against MDR human cancer cells [6,7] and *Leishmania* [8], which was comparable to verapamil. Thus, to continue our research toward the discovery of new DAS as potential Pgp inhibitors, we report herein on the isolation, structure elucidation and bioactivity of thirteen new (**1**–**5** and **9**–**16**) and three known (**6** [9], **7** [10] and **8** [9]) sesquiterpenes with a dihydro-β-agarofuran skeleton from *Maytenus jelskii* Zahlbruchner. Their stereostructures were elucidated by application of 1D and 2D NMR techniques, including COSY, HSQC, HMBC and ROESY experiments, and their absolute configurations were determined by CD studies, chemical correlations or biosynthetic grounds. The isolated compounds have been tested on human MDR1-transfected NIH-3T3 mammalian cells, in order to determine their ability to reverse the MDR phenotype due to Pgp overexpression. All compounds were able to block Pgp transport with low intrinsic cytotoxicity, and eight of them (**1**, **2**, **4**, **6**, **8**, **9**, **11** and **14**) showed an effectiveness that was similar to (or higher than) the classical Pgp inhibitor verapamil for the reversion of resistance to DNM and VNB. The structure–activity relationships are discussed.

2. Results and discussion

2.1. Chemistry

The hexane–Et_2O extract of the fruits of *M. jelskii* was subjected to multiple chromatographic steps, involving vacuum liquid chromatography (VLC), medium-pressure liquid chromatography (MPLC) and preparative TLC on silica gel and Sephadex LH-20 to yield 16 sesquiterpene esters (**1**–**16**, Scheme 1). The structures of the new compounds, **1**–**5** and **9**–**16**, were deduced as described.

Compound **1** was obtained as a colorless lacquer and gave the molecular formula $C_{38}H_{40}O_{13}$ by HRESIMS. The IR spectrum showed absorption bands for hydroxy (3547 cm^{-1}), ester (1746, 1720 cm^{-1}) and aromatic (759, 713 cm^{-1}) groups. The mass spectrum exhibited peaks attributable to the presence of methyl ($[M-15]^+$, m/z 689), benzoate ($[M-122]^+$, m/z 582 and $[M-2\times122]^+$, m/z 460), furoate ($[M-460-112]^+$, m/z 348) and acetate ($[M-122-60]^+$, m/z 522) groups. This was confirmed by the ^1H and ^{13}C NMR data, which included signals of thirteen aromatic protons (δ_H 6.78–8.19), sixteen aromatic carbons (δ_C 110.1–148.6) and three carboxyl groups at δ_C 161.7, 165.7 and 166.0, assigned to one furoyl and two benzoyl moieties, in addition to signals corresponding to two acetyl groups [δ_H 1.73 (s), δ_C 20.1 (q), 169.5 (s) and δ_H 2.36 (s), δ_C 20.5 (q), 170.7 (s)]. Its ^1H NMR spectrum (Table 1) displayed signals for two methylene systems at δ 2.27 (2H, m) and 2.32 (1H, m), 2.72 (1H, ddd, $J=3.5$, 7.0, 15.9 Hz) and one oxymethylene system at δ 4.52, 5.31 (2H, d_{AM}, $J=12.8$ Hz), assigned to protons H_2-3, H_2-8 and H_2-15, respectively. Resonances for four oxymethine protons at δ 5.47 (d, $J=7.0$ Hz, H-9), δ 5.70 (d, $J=3.5$ Hz, H-1), δ 5.83 (br d, $J=3.5$ Hz, H-2) and δ 6.33 (s, H-6) and one aliphatic methine proton at δ 2.41 (t, $J=3.5$ Hz, H-7) were also observed. When **1** was treated with acetic anhydride in pyridine, compound **1** was unaltered, this fact together with the presence of a singlet at δ_H 3.12, interchangeable with D_2O, confirmed the presence of a tertiary hydroxy group. A methyl group at δ 1.60 (H_3-14) attached to a carbon at δ 69.8, bearing a hydroxy group, and two methyls at δ 1.63 (H_3-12) and 1.60 (H_3-13) were confirmed from the ^{13}C NMR spectrum (Table 1). All these data indicate that **1** is a polyester sesquiterpene with a 1,2,4,6,9,15-hexasubstituted dihydro-β-agarofuran skeleton.



Scheme 1. Structure of compounds **1**–**16** and chemical correlations.

Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR (δ, $CDCl_3$, J in Hz in parentheses) data of compounds **1–5** and **9**.

No.	**1**		**2**		**3**		**4**		**5**		**9**	
	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1	5.70 d (3.5)	70.2 d	5.54 d (3.2)	70.0 d	5.47 d (3.1)	73.2 d	5.55 d (3.1)	73.4 d	5.54 d (3.3)	70.3 d	5.58 d (3.4)	70.1 d
2	5.83 br d (3.5)	68.8 d	5.50 m	68.3 d	4.33 m	67.3 d	4.40 m	67.4 d	5.51 dd (3.3, 6.6)	68.1 d	5.84 br d (3.4)	69.4 d
3	2.27 m	42.4 t	α 2.04 d (15.0); β 2.21 br d (15.0)	42.4 t	2.10 br d (3.1)	44.2 t	2.17 m	44.3 t	α 2.06 dd (3.3, 15.4); β 2.16 m	41.9 t	α 2.22 dd (3.2, 15.2); β 2.32 d (15.2)	42.5 t
4	3.12-OH s	69.8 s	3.05-OH s	70.5 s	3.01-OH s	70.0 s	3.08-OH s	70.1 s	2.87-OH s	69.7 s	3.22-OH s	70.0 s
5		91.1 s		91.2 s		91.4 s		91.5 s		91.0 s		91.3 s
6	6.33 s	78.6 d	6.25 s	78.8 d	6.29 s	78.8 d	6.32 s	78.8 d	6.14 s	78.1 d	5.83 s	80.0 d
7	2.41 t (3.5)	49.2 d	2.36 m	49.2 d	2.34 t (3.6)	49.0 d	2.41 t (4.4)	49.1 d	2.22 t (4.7)	49.1 d	2.42 t (3.2)	49.0 d
8	β 2.32 m; α 2.72 ddd (3.5,7.0, 15.9)	35.6 t	β 2.27 br d (15.8); α 2.67 ddd (3.5, 7.0, 15.8)	34.8 t	β 2.25 br d (15.9); α 2.65 ddd (3.6, 6.8, 15.9)	34.7 t	β 2.34 m; α 2.72 ddd (4.4, 7.2, 16.1)	34.7 t	β 2.21 dd (4.7, 16.9); α 2.56 ddd (4.7, 7.2, 16.9)	34.7 t	β 2.27 m; α 2.64 ddd (3.2, 6.8, 16.5)	31.3 t
9	5.47 d (7.0)	68.4 d	5.37 d (7.0)	68.5 d	5.37 d (6.8)	68.8 d	5.52 d (7.2)	69.4 d	5.35 d (7.2)	68.4 d	5.00 d (6.8)	72.1 d
10		54.8 s		54.9 s		55.0 s		55.2 s		54.8 s		51.1 s
11		84.7 s		84.8 s		84.3 s		84.5 s		84.5 s		84.8 s
12	1.63 s	29.4 q	1.57 s	29.6 q	1.56 s	29.5 q	1.62 s	29.5 q	1.58 s	29.4 q	1.60 s	29.7 q
13	1.60[b] s	25.5 q	1.54 s	25.7 q	1.53 s	25.6 q	1.58 s	25.8 q	1.54 s	25.6 q	1.56 s	25.7 q
14	1.60[b] s	25.2 q	1.50 s	24.9 q	1.60 s	25.0 q	1.61 s	25.1 q	1.52 s	25.1 q	1.66 s	25.4 q
15	4.52, 5.31 d_{AM} (12.8)	66.0 t	4.41, 4.96 d_{AM} (13.0)	65.6 t	4.56, 5.02 d_{AM} (13.1)	65.9 t	4.63, 5.06 d_{AM} (13.1)	66.0 t	4.38, 4.99 d_{AM} (12.9)	65.5 t	1.69 s	21.8 q

[a] Data are based on DEPT, HSQC, and HMBC experiments.
[b] Overlapping signals.

The regiosubstitution of **1** was determined by an HMBC experiment, showing three-bond correlations between the carboxyl signals of the acetate groups at δ_C 169.5 and δ_C 170.7 and the resonances at δ_H 5.70 (H-1) and δ_H 4.52, 5.31 (H_2-15), respectively. The carboxyl signal of the furoate group at δ_C 161.7 was correlated with the signal at δ_H 5.47 (H-9), whereas the carboxyl signal of the benzoate groups at δ_C 165.7 and δ_C 166.0 were linked to the resonance at δ_H 6.33 (H-6) and δ_H 5.83 (H-2), respectively. The relative stereochemistry of **1** was established on the basis of the coupling constants and confirmed by a ROESY experiment. Therefore, the $J_{1,2}$ (3.5 Hz) indicated a *cis* relationship between H-1/H-2, and ROE effects of H_2-15 to H-6, H-9 and H_3-14, and those of H-1 to H-2 and H-3β were observed in the ROESY experiment (Fig. 1). The absolute configuration of **1** was resolved by the dibenzoate chirality method, an extension of the circular dichroism exciton chirality procedure [11]. The angle (57.3°) between the two chromophores (benzoate groups) was calculated by molecular mechanic calculations using the PC model [12]. Therefore, compound **1** was considered suitable for a CD study, showing a Davidoff-type split curve with a first negative Cotton effect at 235.7 nm ($\Delta\varepsilon$ −8.3) and a second positive effect at 221.3 nm ($\Delta\varepsilon$ +5.7), due to the couplings of the two benzoate groups at C-2α and C-6β (Fig. 1). Thus, the absolute configuration of **1** was established as (1*R*,2*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-1,15-diacetoxy-2,6-dibenzoyloxy-9-(3-furoiloxy)-4-hydroxy-dihydro-β-agarofuran.

Compound **2** showed a molecular formula of $C_{33}H_{38}O_{13}$ by HRESIMS. The ^{1}H and ^{13}C NMR data (Table 1) showed that it had similar features to those of compound **1**, with the only difference being the replacement of the benzoate group at C-2 in **1** by an acetate group in **2**. 2D NMR (COSY, ROESY, HSQC and HMBC) experiments allowed the complete and unambiguous assignment of the chemical shifts, regiosubstitution and relative configuration of compound **2**. The HRESIMS of compound **3** gave a molecular formula of $C_{31}H_{36}O_{12}$. Its ^{1}H and ^{13}C NMR data (Table 1) were closely related to those of **2**, the main difference was that the resonances assigned to the acetate group at C-2 in **2** were replaced by those for a hydroxy group in **3**, together with the shift of the signal corresponding to the H-2 from δ_H 5.50 in **2** to δ_H 4.33 in **3**. These data suggested that **3** was the 2-deacetyl derivative of **2**, this was supported by 2D NMR analysis, which verified the 1D NMR data, relative configuration and regiosubstitution assignments. The absolute configuration of **2** and **3** was established by chemical correlation. Thus, benzoylation and acetylation of **3** yielded compounds whose spectroscopic data were identical to those of **1** and **2**, respectively (Scheme 1).

Compounds **4** and **5** showed spectroscopic data similar to **1**–**3** and to the other known isolated compounds (**6**–**8**) [9,10]. Consequently, the stereostructure elucidation of these molecules was greatly aided by comparison of their spectroscopic data. Even so, it should be noted that a complete set of 2D NMR spectra (COSY, HSQC and HMBC) was acquired for each metabolite in order to gain the

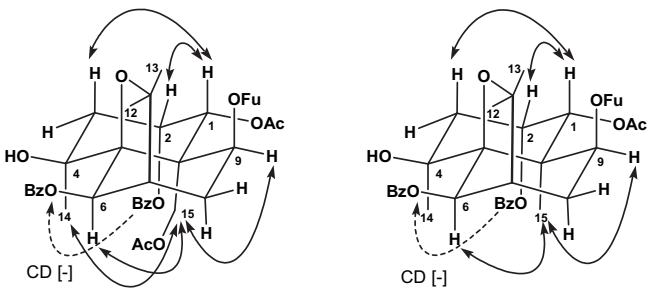


Fig. 1. ROE effects (solid line) and CD exciton coupling (dashed line) for compounds **1** (left) and **9** (right).

complete and unambiguous assignment of the ^{1}H and ^{13}C NMR resonances as listed in Table 1. The same relative stereochemistry previously assigned to **1** was also confirmed for **4** and **5** by 2D NMR ROESY experiments, and their absolute configurations were assumed based on their having the same polyhydroxy sesquiterpene core as compound **1**.

Compound **9**, with a molecular formula of $C_{36}H_{38}O_{11}$ (HRESIMS), was revealed to be the 15-deacetoxy derivative of **1** by analysis of its 1D and 2D NMR spectra (Table 1 and Experimental section, Fig. 1). Thus, comparison of their NMR data showed the replacement of the resonances for an acetate group at C-15 (δ_H 4.52, 5.31, δ_C 66.0) in **1** by resonances for a methyl group (δ_H 1.69, δ_C 21.8) in **9**, along with the $^{1}H-^{13}C$ long-range correlations for Me-15/C-1, C-5, C-9 and C-10 observed in the HMBC experiment. The absolute configuration of **9** was resolved by a circular dichroism study [11], showing a Davidoff-type split curve with a first negative Cotton effect at 235.5 nm ($\Delta\varepsilon$ −7.6) and a second positive effect at 221.6 nm ($\Delta\varepsilon$ +6.3), due to the couplings of the two benzoate groups at C-2α and C-6β (Fig. 1). Therefore, the absolute configuration of **9** was established as (1*R*,2*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-1-acetoxy-2,6-dibenzoyloxy-9-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran.

Compound **10** was obtained as a colorless lacquer. The ^{1}H and ^{13}C NMR spectra (Table 2) combined with the mass data were used to established its structure as that of a 1,2,3,4,9-pentasubstituted dihydro-β-agarofuran sesquiterpene polyester containing two acetate, one benzoate, one furoate and one hydroxy groups. The structure and NMR data assignments of **10** were proved by the 2D NMR data. Particularly, the regiosubstitution was determined by the long-range $^{1}H-^{13}C$ HMBC couplings observed between the H-1 (δ_H 6.18), H-2 (δ_H 5.19), H-3 (δ_H 5.24) and H-9 (δ_H 5.02) proton resonances and the carboxyl signals of the furoate, (δ_C 160.8) acetate (δ_C 170.1 and 170.2) and benzoate (δ_C 165.2) groups, respectively. The relative configuration was established on the basis of the coupling constants and confirmed by a ROESY experiment (Fig. 2). Therefore, the $J_{1,2}$ (10.8 Hz) and $J_{2,3}$ (2.8 Hz) values indicated a *trans* and *cis* relationship between H-1/H-2 and H-2/H-3, respectively. The ROESY experiment showed ROE effects of H-2 to H_3-14 and H_3-15, those of H-3 to H_3-14 and H-9 to H_3-15. The absolute configuration of **10** was assumed based on it having the same polyhydroxy core as another sesquiterpene previously isolated from the leaves of *M. jelskii* and whose absolute configuration has been established by a CD study [13].

The molecular formula $C_{31}H_{36}O_{10}$ of **11** indicated one fewer oxygen atoms than **10**. Comparison of their NMR data (Table 2 and Experimental section, Fig. 2) showed that the resonances of the characteristic tertiary hydroxy group at C-4 (δ_H 3.43 interchangeable with D_2O, δ_C 69.7) in **10** were replaced by those of an aliphatic methine group (δ_H 2.41, δ_C 43.4) in **11**. These data suggested that **11** was the 4-deoxy-derivative of **10**. The HREIMS of compound **12** gave a molecular ion at *m*/*z* 526.2185, corresponding to a molecular formula of $C_{29}H_{34}O_9$. Its ^{1}H and ^{13}C NMR data (Table 2) were closely related to those of **10**, the predominant differences being the replacement of the resonances corresponding to the acetate group on C-3 by those of an aliphatic methylene (δ_H 2.09, δ_C 42.7) in **12**. These data suggested that **12** was the 3-deacetoxy derivative of **10**. Compounds **13** and **14** gave molecular formulas of $C_{27}H_{32}O_{10}$ and $C_{29}H_{34}O_9$, respectively, by HREIMS. Their ^{1}H and ^{13}C NMR data (Table 2) showed the same 1,2,4,9-tetrasubstituted dihydro-β-agarofuran skeleton as **12**. Consequently, their stereostructural elucidation was greatly aided by the comparison of their spectroscopic data with those for **12**, the only difference being the ester group located at C-9. In fact, the NMR data for compounds **13** and **14** showed signals corresponding to a furoate and a cinnamate group, respectively, at this position. Even, so, it should be noted that a complete set of 2D NMR spectra (COSY, ROESY, HSQC and HMBC)

Table 2
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR (δ, $CDCl_3$, *J* in Hz in parentheses) data of compounds **10**–**16**.

No.	**10**		**11**		**12**		**13**		**14**		**15**		**16**	
	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1	6.18 d (10.8)	67.3 d	6.42 d (11.1)	68.4 d	5.84 d (10.5)	72.6 d	5.75 d (10.3)	72.3 d	5.83 d (10.6)	72.5 d	5.93 d (10.2)	73.4 d	5.53 dd (3.5, 11.9)	73.2 d
2	5.19 dd (2.8, 10.8)	68.6 d	5.42 dd (3.0, 11.1)	67.9 d	5.11dt (4.8, 10.5)	69.1 d	5.06 dt (4.1, 10.3)	68.9 d	5.9 dt (4.7, 10.6)	69.2 d	5.29 dt (4.7, 10.2)	69.3 d	α 1.56 br d (11.9); β 2.09 m	23.8 t
3	5.24 d (2.8)	75.5 d	5.00 d (3.0)	75.0 d	2.08 m	42.7 t	2.08 m	42.4 t	2.09 m	42.6 t	α 1.84 br d (12.8); β 2.37 dt (4.7, 12.8)	33.1 t	α 1.77 dt (3.4, 13.4); β 1.94 dt (3.8, 13.4)	37.0 t
4	3.43-OH s	69.7 s	2.41 q (8.1)	43.4 d	2.91-OH s	70.6 s	2.84-OH s	70.3 s	2.86-OH s	70.5 s	2.10 m	40.7 d	2.74-OH s	70.3 s
5		89.5 s		85.8 s		90.0 s		89.7 s		89.9 s		86.9 s		90.6 s
6	α 1.94 d_{AB} (12.4); β 2.44 dd_{AB} (3.3, 12.4)	32.5 t	α 2.17 m; β 2.06 m	36.2 t	α 1.84 d (1.4); β 2.47 br d (12.4)	31.6 t	α 1.81 d (12.4); β 2.43 br d (12.4)	31.4 t	α 1.80 d (12.5); β 2.43 br d (12.5)	31.5 t	α 2.05 m; β 2.18[b] m	36.3 t	α 1.82 d (12.3); β 2.44 br d (12.3)	31.6 t
7	2.09 m	42.5 d	2.04 m	42.6 d	2.09[b] m	43.4 d	2.03 m	43.2 d	2.09 m	43.4 d	2.06 m	43.9 d	2.10 m	43.5 d
8	2.18 m	30.0 t	2.13 m	30.3 t	α 2.23 m; β 2.09[b] m	31.0 t	2.12 m	30.8 t	2.14 m	31.0 t	α 2.18[b] m; β 2.08 m	31.4 t	α 2.25 ddd (3.4, 6.6, 16.0); β 2.12 br d (16.0)	31.2 t
9	5.02 d (5.8)	72.9 d	4.98 d (5.3)	73.3 d	5.05 d (6.5)	73.6 d	4.91 d (6.8)	72.9 d	4.76 d (5.0)	73.4 d	4.97 d (6.4)	74.0 d	5.10 d (6.6)	74.0 d
10		47.4 s		49.4 s		48.3 s		47.9 s		48.2 s		49.6 s		48.1 s
11		83.9 s		82.3 s		84.1 s		83.7 s		83.9 s		82.6 s		83.5 s
12	1.31 s	29.7 q	1.18 s	30.0 q	1.36 s	30.0 q	1.33 s	29.8 q	1.33 s	30.1 q	1.26 s	30.2 q	1.35 s	30.0 q
13	1.43 s	24.2 q	1.34 s	24.2 q	1.48 s	24.4 q	1.45 s	24.1 q	1.45 s	24.2 q	1.41 s	24.4 q	1.47 s	24.4 q
14	1.45 s	23.7 q	1.27 d (8.1)	15.9 q	1.42 s	25.0 q	1.39 s	24.7 q	1.39[b] s	24.9 q	1.25 d (7.6)	18.5 q	1.31 s	24.1 q
15	1.42 s	19.6 q	1.41 s	18.9 q	1.45 s	20.0 q	1.40 s	19.7 q	1.39[b] s	19.8 q	1.42 s	19.3 q	1.37 s	19.3 q

[a] Data are based on DEPT, HSQC, and HMBC experiments.
[b] Overlapping signals.

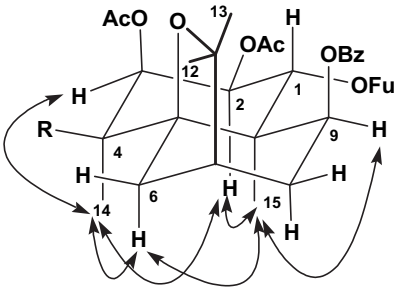


Fig. 2. ROE effects for compounds **10** and **11**.

was acquired for each metabolite in order to gain the complete and unambiguous assignment of the ^{1}H and ^{13}C NMR resonances as listed in Table 2.

The molecular formula $C_{29}H_{34}O_8$ of compound **15** (HREIMS) had one fewer oxygen atom than **12**. Comparison of their NMR data (Table 2 and Experimental section) showed that the resonances of the characteristic tertiary hydroxyl group at C-4 (δ_H 2.91, interchangeable with D_2O; δ_C 70.6) in **12** were replaced by those of an aliphatic methine group (δ_H 2.10, δ_C 40.7) in **15**. These data suggested that **15** was the 4-deoxy-derivative of **12**, proposal supported by combined analysis of 1D and 2D spectra. The HREIMS of compounds **16** gave a molecular ion at *m/z* 468.2129, corresponding to a molecular formula $C_{27}H_{32}O_7$. Its ^{1}H and ^{13}C NMR data (Table 2) were closely related to those of **12**, except for the absence of the signals assigned to the acetate group at C-2 and the shift of the signals corresponding to the C-2 position from δ_H 5.11 and δ_C 69.1 in **12** to δ_H 1.56, 2.09 and δ_C 23.8 in **16**. These data indicated that **16** was the 3-deacetoxy derivative of **12**. Detailed assignments of the ^{1}H and ^{13}C NMR data and its relative configuration were determined based on 2D spectra analysis.

The absolute configuration of compounds **11**–**16** could be assumed based on biosynthetic considerations. We propose that the biosynthetic pathway involves stereospecific introduction of hydroxyl groups while retaining the configuration in the polyhydroxy sesquiterpene core. Thus, compounds **15** and **16** could be considered early precursors to the more functionalized basic polyhydroxy sesquiterpene core of compound **10**, whose absolute configuration was previously established. Compounds **11**–**14** are also likely to be biosynthetic intermediates of compound **10**. The new compounds have the basic polyhydroxy-dihydro-β-agarofuran sesquiterpenoid core of 3-deoxymaytol [14] (**1**–**5**), 2α,4β-dihydroxycelorbicol [15] (**9**), 6-deoxymagellanol [16] (**10**), 4,6-dideoxymagellanol [16] (**11**), 3,6-dideoxymagellanol [16] (**12**–**14**), 2-*epi*-isocelorbicol [15] (**15**) and 4β-hydroxy-6-deoxycelorbicol [15] (**16**).

2.2. Biological evaluation

The biological activity of the isolated sesquiterpenes (**1**–**16**) was monitored through their ability to inhibit Pgp-mediated DNM efflux and to reverse cellular MDR in NIH-3T3 MDR1G-185 mammalian cells (MDR1 cells). Flow cytometry analysis shows that all of them demonstrated a Pgp inhibitory activity at 10 μM, and the eight most active compounds were able to block up to 70% of DNM transport activity of the protein (Fig. 3). Afterward, the K_i (concentration that inhibits DNM transport by 50%) was determined with values of 0.34 ± 0.09, 0.59 ± 0.16, 0.47 ± 0.09, 0.39 ± 0.05, 0.51 ± 0.04, 0.38 ± 0.10, 0.64 ± 0.16, and 0.57 ± 0.18 μM for compounds **1**, **2**, **4**, **6**, **8**, **9**, **11** and **14**, respectively. These eight sesquiterpenes possess I_{max} values (maximal percentage inhibition of DNM transport) higher than 90% (data not shown). This efficiency in blocking DNM transport was correlated with their ability to sensitize MDR1 cells to the cytotoxic drugs DNM and VNB. In this regard, the eight most efficient sesquiterpenes in blocking DNM efflux were able to reverse DNM and VNB resistance in MDR1 cells with reversal index values comparable to or higher than the classical Pgp inhibitor verapamil (Table 3).

These sesquiterpenes showed similar toxicity to verapamil for both wild-type and MDR1 cells (Table 4). One conclusion can be drawn from these results: Pgp does not confer cross-resistance to sesquiterpenes or, in other words, the sesquiterpenes are either poor Pgp substrates or not transported at all by the Pgp. Similar results were found for other sesquiterpenes in our previous work [7].

2.3. SAR analysis

The influence of the substitution pattern of the dihydro-β-agarofuran skeleton on the MDR reversal activity of the

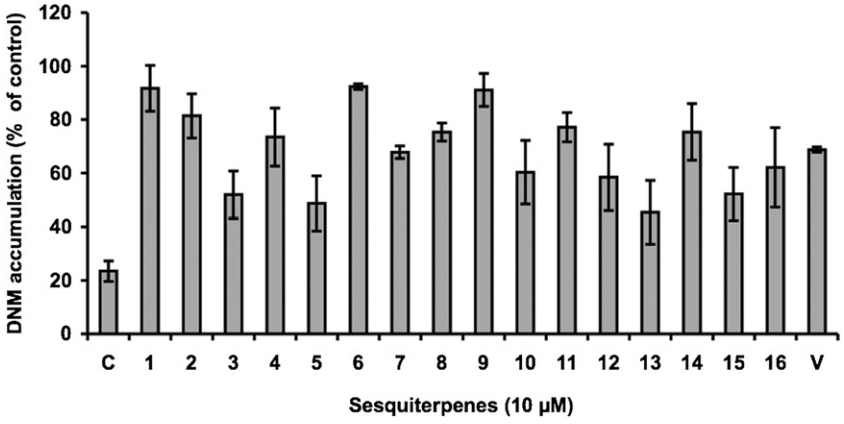


Fig. 3. Inhibition of Pgp-mediated DNM transport across the cell membrane in intact mammalian MDR1 cells. The cells were co-incubated with 2 μM DNM and 10 μM of sesquiterpenes, the DNM retention was determined by flow cytometry analysis as described in Biological assays. DNM fluorescence intensity is expressed as relative fluorescence compared to a control (5 mM ortho-vanadate), representing 100% inhibition of Pgp. C = MDR1 cells incubated with DNM and without compounds. V = 10 μM verapamil, a classical Pgp reversal agent used for comparison. Data are the mean ± S.D. of three independent experiments performed in triplicate.

Table 3
Drug resistance reversal ability of compounds in MDR1 cells.[a]

Compound	Reversal index with DNM[b]			Reversal index with VNB[c]		
	1 μM	3 μM	10 μM	1 μM	3 μM	10 μM
1	7.82 ± 2.79	14.07 ± 2.82	17.11 ± 3.68	10.34 ± 4.41	14.26 ± 4.43	51.67 ± 2.69
2	4.50 ± 0.80	11.97 ± 3.67	13.27 ± 2.33	7.51 ± 1.54	13.21 ± 2.77	58.13 ± 5.41
4	2.21 ± 1.02	6.25 ± 1.54	8.82 ± 2.57	2.55 ± 1.15	13.26 ± 0.80	36.60 ± 4.36
6	12.73 ± 1.39	19.80 ± 3.22	21.24 ± 3.09	28.60 ± 3.44	52.56 ± 2.06	165.10 ± 5.82
8	2.72 ± 0.62	4.37 ± 1.13	14.63 ± 0.17	3.58 ± 1.09	18.85 ± 2.54	65.64 ± 2.07
9	4.36 ± 1.04	10.35 ± 1.68	33.97 ± 4.21	11.14 ± 1.81	30.32 ± 5.77	125.31 ± 6.25
11	1.93 ± 0.38	3.68 ± 0.72	10.44 ± 1.85	3.50 ± 0.71	22.36 ± 2.46	73.92 ± 5.07
14	1.59 ± 0.44	7.77 ± 1.78	16.57 ± 2.60	4.89 ± 1.21	19.90 ± 1.16	83.66 ± 3.88
Verapamil	3.04 ± 0.57	8.79 ± 2.35	10.64 ± 1.97	6 .23 ± 0.56	20.85 ± 2.84	76.81 ± 7.01

[a] The reversal index is the fined as the ratio between the IC_{50} of cells without compound and the IC_{50} with compound. IC_{50} values were determined as described in the Biological assays section. Results are expressed as the mean ± S.D. of three independent experiments performed in triplicate.
[b] The maximum reversal index with DNM (ratio between IC_{50} for MDR1 and parental drug-sensitive cells) was 55.
[c] The maximum reversal index with VNB (ratio between IC_{50} for MDR1 and parental drug-sensitive cells) was 250.

sesquiterpenes studied was examined, revealing the following trends on the preliminary structure–activity relationship of this series of natural sesquiterpenes. In general, the more active compounds against DNM present an ester group at C-6 (compounds **1**–**9**). Compounds with an aromatic ester group (benzoate or furoate) at C-6 are more active than those with an acetate group in such position (**6** and **8** *versus* **5** and **7**, respectively). Results showed a reversal activity sequence of cinnamate > benzoate > furoate groups at C-9 (**14** *versus* **12** and **13**; **6** *versus* **2**). Furthermore, an acetate group at C-3 (**10** *versus* **12**) or C-15 (**2** *versus* **9**) reduces the reversal activity. In addition, the presence of a hydroxyl group at C-2 produces a decrease in activity (**3** *versus* **1** and **2**; **4** *versus* **6**). No straightforward conclusion can be drawn from the presence/absence of the hydroxy group at C-4β, given that its presence (**11** *versus* **10**; **12** *versus* **15**) decreases or increases, respectively, the DNM accumulation in MDR1 cells. Similar correlation was found for the VNB reversal activity, especially when the analysis is performed with lower concentrations of sesquiterpenes. These trends, based upon substitution patterns, are consistent with our previous SAR findings [6–8], and reinforce our statement that the overall esterification level of the molecule and the presence of aromatic ester groups seems to be responsible for the potent MDR reversal activity of this type of metabolites.

3. Conclusion

A new series of dihydro-β-agarofuran sesquiterpenes isolated from *M. jelskii* have been evaluated as reversal agents of the MDR phenotype in mammalian cells. Eight of the isolated sesquiterpenes showed a potent reversal activity of the MDR phenotype mediated by the human Pgp overexpression. Compounds **6** and **9** were the most active agents, reversing both DNM and VBN resistance and showed higher activity than the reference reversal agent verapamil. Structure–activity relationships among sesquiterpenes showed that the overall esterification level of the compound and the presence of aromatic ester groups at C-6 and C-9 seems to be an important element to enhance the MDR reversal activity, findings that are consistent with our previous studies. The elucidation of the pharmacophore will be helpful for the rational design of more potent and specific Pgp inhibitors with potential use in the treatment of MDR cancers.

Table 4
Intrinsic toxicity of sesquiterpenes in parental and MDR1 cells.[a]

Compound	Parenteral cells	MDR1 cells
1	82.14 ± 1.62	84.33 ± 2.65
2	92.36 ± 5.76	87.84 ± 0.18
4	84.38 ± 4.43	104.77 ± 13.21
6	76.70 ± 1.61	77.21 ± 4.23
8	91.98 ± 5.83	82.86 ± 2.45
9	108.24 ± 3.56	98.02 ± 1.84
11	91.78 ± 8.12	92.37 ± 5.82
14	85.95 ± 4.13	100.10 ± 0.12
Verapamil	102.91 ± 3.32	79.18 ± 8.93

[a] Toxicity was determined using 10 μM of compounds. Results are expressed as percentage mean of cell growth ± S.D. with respect to the control without compounds. Data are the mean of three independent experiments performed in triplicate.

4. Experimental

4.1. General

Optical rotations were measured on a Perkin Elmer 241 automatic polarimeter in $CHCl_3$ at 25 °C and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. UV spectra were obtained in absolute EtOH on a JASCO V-560 instrument. IR (film) spectra were measured in $CHCl_3$ on a Bruker IFS 55 spectrophotometer. ^{1}H (300 and 400 MHz) and ^{13}C (75 and 100 MHz) NMR spectra were recorded on a Bruker Avance 300 and 400 spectrometers; chemical shifts were referred to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0); DEPT, COSY, ROESY (spin lock field 2500 Hz), HSQC and HMBC (optimized for $J = 7.7$ Hz) experiments were carried out with the pulse sequences given by Bruker. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer, and HRESIMS (positive mode) were measured on an LCT Premier XE Micromass Electrospray spectrometer. Silica gel 60 (15–40 μm) for column chromatography, silica gel 60 F_{254} for TLC, and nanosilica gel 60 F_{254} for preparative HPTLC were purchased from Macherey-Nagel, and Sephadex LH-20 was obtained from Pharmacia Biotech. The spots were visualized by UV light and heating silica gel plates sprayed with H_2O–H_2SO_4–AcH (1:4:20). All solvents used were analytical grade from Panreac. Reagents were purchased from Sigma–Aldrich and used without further purification. Compounds used for CD were purified by high-performance TLC and eluted with a mixture of *n*-hexane–EtOAc.

4.2. Plant material

M. jelskii was collected in the Urubamba Privince, Cuzco, Perú, in December 2006. A voucher specimen (CUZ 29845) was identified by Professor Alfredo Tupayachi Herrera and deposited in the Herbarium of Missouri Botanical Garden, St. Louis, MO.

4.3. Extraction and isolation

The fruits (250 g) of *M. jelskii* were extracted three times with *n*-hexane–Et_2O (1:1) for 24 h, each time in a Soxhlet apparatus. Removal of the solvent under reduced pressure provided 42 g of crude extract, which was chromatographed by dry flash chromatography on a silica gel column, using mixtures of *n*-hexane–EtOAc of increasing polarity to afford 30 fractions which were combined in six fractions (A–F) on the basis of their TLC profiles. Preliminary NMR studies revealed that four fractions (C–F) were found to be rich in dihydro-β-agarofuran sesquiterpenoids and were further investigated. These fractions were purified after several chromatographies on Sephadex LH-20 using *n*-hexane–$CHCl_3$–MeOH (2:1:1) as eluant. The obtained fractions were chromatographed by wet flash chromatography on a silica gel column, using mixtures of CH_2Cl_2–Me_2CO of increasing polarity, and further purified by preparative TLC with *n*-hexane–dioxane (6:4), *n*-hexane–Et_2O (2:8) or CH_2Cl_2–Me_2CO (8:2) to give the compounds. Following this procedure, fraction C led to the isolation of compounds **7** (2.7 mg), **9** (32.5 mg), **15** (5.0 mg) and **16** (8.0 mg). In the same way, fraction D afforded compounds **1** (40.8 mg), **2** (225.7 mg), **6** (25.8 mg) and **11** (14.6 mg). Fraction E gave compounds **8** (3.4 mg), **10** (40.5 mg), **12** (18.6 mg), **13** (3.1 mg) and **14** (78.6 mg). Finally, fraction F yielded compounds **3** (23.2 mg), **4** (1.3 mg) and **5** (6.5 mg). Compounds **1** and **9**, used for CD studies, were further purified by high-performance TLC, using as eluant *n*-hexane–EtOAc 1:1 and 6:4, respectively.

4.3.1. (1R,2S,4S,5S,6R,7R,9S,10R)-1,15-Diacetoxy-2,6-dibenzoyloxy-9-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**1**)

Colorless laquer, $[\alpha]_D^{25}$ +55.3 (*c* 1.0, $CHCl_3$); CD λ_{ext} (MeCN) 235.7 ($\Delta\varepsilon = -8.3$), 221.3 ($\Delta\varepsilon = +5.7$) nm; UV λ_{max} (EtOH) (log ε) 230 (4.0), 213 (3.8) nm; IR ν_{max} (film) 3547, 2928, 1746, 1720, 1273, 1223, 1108, 1023, 759, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.73 (3H, s, OAc-1), 2.36 (3H, s, OAc-15), OFu-9 [6.78 (1H, s, H-4), 7.49 (1H, m, H-5), 8.07 (1H, s, H-2)], OBz-6 [7.50 (2H, m, H-3, H-5), 7.60 (1H, m, H-4), 8.14 (2H, d, $J = 7.9$ Hz, H-2, H-6)], OBz-2 [7.50 (2H, m, H-3, H-5), 7.60 (1H, m, H-4), 8.19 (2H, d, $J = 7.9$ Hz, H-2, H-6)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OFu-9 [110.1 (d, C-4), 118.5 (s, C-3), 143.8 (d, C-5), 148.6 (d, C-2), 161.7 (s, COO–)], OBz-6 [128.6 (2 × d, C-3, C-5), 129.3 (s, C-1), 129.8 (d, C-2), 129.9 (d, C-6), 133.4 (d, C-4), 165.7 (s, COO–)], OBz-2 [128.7 (2 × d, C-3, C-5), 129.7 (s, C-1), 130.0 (d, C-2), 130.2 (d, C-6), 133.5 (d, C-4), 166.0 (s, COO–)], OAc [(20.1 q, 169.5 s, OAc-1), (20.5 q, 170.7 s, OAc-15)], for other signals, see Table 1; EIMS *m/z* 689 $[M - CH_3]^+$ (1), 582 (1), 522 (1), 460 (22), 348 (1), 275 (7), 192 (7), 105 (100), HREIMS *m/z* 689.2234 $[M - CH_3]^+$ (calcd for $C_{37}H_{37}O_{13}$, 689.2241); HRESIMS *m/z*: 727.2352 $[M + Na]^+$ (calcd for $C_{38}H_{40}O_{13}Na$, 727.2367).

4.3.2. (1R,2S,4S,5S,6R,7R,9S,10R)-1,2,15-Triacetoxy-6-benzoyloxy-9-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**2**)

Colorless laquer; $[\alpha]_D^{25}$ +40.5 (*c* 1.1, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 231 (3.9), 213 (3.7) nm; IR ν_{max} (film) 3548, 2956, 2932, 1747, 1723, 1575, 1278, 1242, 1155, 758, 715 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.71 (3H, s, OAc-1), 2.10 (3H, s, OAc-2), 2.31 (3H, s, OAc-15), OFu-9 [6.74 (1H, d, $J = 0.7$ Hz, H-4), 7.45 (1H, m, H-5), 8.02 (1H, d, $J = 0.7$ Hz, H-2)], OBz-6 [7.46 (2H, m, H-3, H-5), 7.57 (1H, m, H-4), 8.18 (2H, d, $J = 8.1$ Hz, H-2, H-6)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OFu-9 [109.9 (d, C-4), 118.6 (s, C-3), 144.0 (d, C-5), 148.9 (d, C-2), 161.9 (s, COO–)], OBz-6 [128.8 (2 × d, C-3, C-5), 129.9 (s, C-1), 130.2 (2 × d, C-2, C-6), 133.5 (d, C-4), 166.1 (s, COO–)], OAc [(20.6 q, 169.6 s, OAc-1), (21.3 q, 169.8 s, OAc-2), (21.5 q, 170.7 s, OAc-15)], for other signals, see Table 1; EIMS *m/z* 627 $[M - CH_3]^+$ (3), 582 (1), 515 (1), 460 (22), 418 (1), 348 (1), 275 (7), 192 (7), 105 (100), 95 (31); HREIMS *m/z* 627.2087 $[M - CH_3]^+$ (calcd for $C_{32}H_{35}O_{13}$, 627.2078); HRESIMS *m/z* 665.2202 $[M + Na]^+$ (calcd for $C_{33}H_{38}O_{13}Na$, 665.2210).

4.3.3. (1R,2S,4S,5S,6R,7R,9S,10R)-1,15-Diacetoxy-6-benzoyloxy-9-(3-furoyloxy)-2,4-dihydroxy-dihydro-β-agarofuran (**3**)

Colorless laquer; $[\alpha]_D^{25}$ +31.3 (*c* 0.4, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 230 (3.3), 218 (3.1) nm; IR ν_{max} (film) 3516, 2926, 2853, 1717, 1278, 1234, 1141, 762, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.80 (3H, s, OAc-1), 2.30 (3H, s, OAc-15), OFu [6.75 (1H, d, $J = 1.7$ Hz, H-4), 7.45 (1H, m, H-5), 8.03 (1H, d, $J = 1.7$ Hz, H-2)], OBz-6 [7.46 (2H, m, H-3, H-5), 7.58 (1H, m, H-4), 8.19 (2H, d, $J = 7.0$ Hz, H-2, H-6)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OFu-9 [109.8 (d, C-4), 118.6 (s, C-3), 143.8 (d, C-5), 148.7 (d, C-2), 161.8 (s, COO–)], OBz-6 [128.6 (2 × d, C-3, C-5), 129.9 (s, C-1), 130.1 (2 × d, C-2, C-6), 133.3 (d, C-4), 166.6 (s, COO–)], OAc [(20.8 q, 169.9 s, OAc-1), (21.3 q, 170.6 s, OAc-15)], for other signals, see Table 1; ESIMS *m/z* 623 $[M + Na]^+$ (100); HRESIMS *m/z* 623.2098 $[M + Na]^+$ (calcd for $C_{31}H_{36}O_{12}Na$, 623.2104).

4.3.4. Benzoylation of **3**

A mixture of benzoyl chloride (4 drops), compound **3** (3.2 mg), 4-(dimethylamino)-pyridine (2.0 mg), and imidazole (2.0 mL) in dry pyridine (2.0 mL) was heated under reflux for 16 h. The mixture was evaporated to dryness and the residue was dissolved in Et_2O and washed with H_2O, 10% $NaHCO_3$, and brine. The organic layer was stirred over KF (64.0 mg) for 1 h and filtered through Celite. The filtrate was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by preparative TLC using *n*-hexane–EtOAc (1:1) to give **1** (2.8 mg).

4.3.5. Acetylation of **3**

A mixture of acetic anhydride (2 drops), triethyl-amine (4 drops), compound **3** (2.9 mg), and 4-(dimethylamino)pyridine in dichloromethane (2 mL) was stirred at room temperature for 16 h. The reaction was quenched by the addition of ethanol (0.5 mL), followed by stirring for 30 min at room temperature. The mixture was evaporated to dryness, and the residue was purified by preparative TLC, using *n*-hexane–EtOAc (1:1) to give compound **2** (2.5 mg).

4.3.6. (1R,2S,4S,5S,6R,7R,9S,10R)-1,15-Diacetoxy-6,9-dibenzoyloxy-2,4-hydroxy-dihydro-β-agarofuran (**4**)

Colorless laquer; $[\alpha]_D^{25}$ +32.7 (*c* 0.1, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 231 (4.1) nm; IR ν_{max} (film) 3521, 2924, 2854, 1746, 1717, 1276, 1107, 1024, 772, 714 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.71 (3H, s, OAc-1), 2.35 (3H, s, OAc-15), OBz-9 [7.50 (2H, m, H-3, H-5), 7.62 (1H, m, H-4), 8.10 (2H, d, $J = 7.4$ Hz, H-2, H-6)], OBz-6 [7.50 (2H, m, H-3, H-5), 7.62 (2H, m, H-4), 8.23 (2H, d, $J = 7.6$ Hz, H-2, H-6)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OBz-9 [128.4 (2 × d, C-3, C-5), 129.1 (s, C-1), 130.1 (2 × d, C-2, C-6), 133.4 (d, C-4), 165.3 (s, COO–)], OBz-6 [128.7 (2 × d, C-3, C-5), 129.9 (s, C-1), 130.2 (2 × d, C-2, C-6), 133.6 (d, C-4), 166.0 (s, COO–)], OAc [(20.7 q, 169.7 s, OAc-1), (21.4 q, 170.6 s, OAc-15)], for other signals, see Table 1; ESIMS *m/z* 633 $[M + Na]^+$ (100); HRESIMS *m/z* 633.2322 $[M + Na]^+$ (calcd for $C_{33}H_{38}O_{11}Na$, 633.2312).

4.3.7. (1R,2S,4S,5S,6R,7R,9S,10R)-1,2,6,15-Tetraacetoxy-9-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**5**)

Colorless laquer; $[\alpha]_D^{25}$ +18.6 (*c* 0.6, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 226 (2.9), 219 (3.8) nm; IR ν_{max} (film) 3541, 2927, 2854, 1746, 1368, 1232, 1154, 1029, 760 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.73 (3H, s, OAc-1), 2.13 (3H, s, OAc-2), 2.14 (3H, s, OAc-6), 2.29 (3H, s, OAc-15), OFu-9 [6.76 (1H, d, $J = 1.6$ Hz, H-4), 7.45 (1H, d, $J = 1.6$ Hz, H-5), 8.04 (1H, s, H-2)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OFu-9 [109.7 (d, C-4), 118.5 (s, C-3), 143.9 (d, C-5), 148.8 (d, C-2), 161.7 (s, COO–)], OAc [(20.5 c, 169.5 s, OAc-1), (21.1 q, 169.7 s, OAc-2), (21.3 q,

170.6 s, OAc-15), (21.6 q, 170.3 s, OAc-6)], for other signals, see Table 1; EIMS *m/z* 565 $[M - CH_3]^+$ (2), 520 (8), 460 (12), 453 (2), 351 (7), 291 (7), 228 (18), 192 (27), 95 (6); HREIMS *m/z* 565.1971 $[M - CH_3]^+$, (calcd for $C_{27}H_{33}O_{13}$ 565.1921); HRESIMS *m/z* 603.2060 $[M + Na]^+$ (calcd for $C_{28}H_{36}O_{13}Na$, 603.2054).

4.3.8. (1R,2S,4S,5S,6R,7R,9S,10R)-1-Acetoxy-2,6-dibenzoyloxy-9-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**9**)

Colorless laquer; $[\alpha]_D^{25}$ +49.5 (*c* 0.4, $CHCl_3$); CD λ_{ext} (MeCN) 235.5 ($\Delta\varepsilon = -7.6$), 221.6 ($\Delta\varepsilon = +6.3$); UV λ_{max} (EtOH) (log ε) 229 (3.4) nm; IR ν_{max} (film) 3546, 2955, 2927, 1719, 1275, 1110, 1024, 759, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.76 (3H, s, OAc-1), OFu-9 [6.76 (1H, d, $J = 0.7$ Hz, H-4), 7.46 (1H, m, H-5), 8.10 (1H, s, H-2)], OBz-2 [7.47 (2H, m, H-3, H-5), 7.60 (1H, m, H-4), 7.99 (2H, d, $J = 8.0$ Hz, H-2, H-6)], OBz-6 [7.50 (2H, m, H-3, H-5), 7.60 (1H, m, H-4), 8.25 (2H, d, $J = 8.0$ Hz, H-2, H-6)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ OFu-9 [109.8 (d, C-4), 118.7 (s, C-3), 143.8 (d, C-5), 148.6 (d, C-2), 161.9 (s, COO−)], OBz-2 [128.6 (2 × d, C-3, C-5), 129.5 (2 × d, C-2, C-6), 129.7 (s, C-1), 133.4 (d, C-4), 165.8 (s, COO−)], OBz-6 [128.7 (2 × d, C-3, C-5), 129.9 (s, C-1), 130.2 (2 × d, C-2, C-6), 133.2 (d, C-4), 166.1 (s, COO−)], OAc (20.5 q, 169.8 s, OAc-1), for other signals, see Table 1; EIMS *m/z* 631 $[M - CH_3]^+$ (1), 613 (1), 519 (2), 402 (12), 290 (6), 230 (5), 105 (100), 95 (23); HREIMS *m/z* 631.2168 $[M - CH_3]^+$, (calcd for $C_{35}H_{35}O_{11}$ 631.2179); HRESIMS *m/z* 669.2283 $[M + Na]^+$ (calcd for $C_{36}H_{38}O_{11}Na$, 669.2312).

4.3.9. (1S,2S,3S,4S,5R,7R,9S,10R)-2,3-Diacetoxy-9-benzoyloxy-1-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**10**)

Colorless laquer; $[\alpha]_D^{25}$ +71.6 (*c* 0.4, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 231 (3.9) nm; IR ν_{max} (film) 3524, 2927, 1745, 1714, 1305, 1245, 1137, 1011, 757, 714 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.77 (3H, s, OAc-2), 2.26 (3H, s, OAc-3), OFu-1 [6.33 (1H, d, $J = 1.9$ Hz, H-4), 7.66 (1H, t, $J = 1.9$ Hz, H-5), 7.31 (1H, d, $J = 1.9$ Hz, H-2)], OBz-9 [7.45 (2H, m, H-3, H-5)], 7.58 (1H, m, H-4), 8.01 (2H, d, $J = 7.7$ Hz, H-2, H-6)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.2 (d, C-4), 118.7 (s, C-3), 143.3 (d, C-5), 147.1 (d, C-2), 160.8 (s, COO−)], OBz-9 [127.7 (2 × d, C-3, C-5), 129.7 (s, C-1), 129.9 (2 × d, C-2, C-6), 132.7 (d, C-4), 165.2 (s, COO−)], OAc [(20.4 q, 170.1 s, OAc-2), (20.8 q, 170.2 s, OAc-3)], for other signals, see Table 2; EIMS *m/z* 584 $[M]^+$ (4), 569 (28), 524 (1), 447 (18), 430 (4), 370 (10), 248 (18), 105 (100), 95 (94); HREIMS *m/z* 584.2266 $[M]^+$ (calcd for $C_{31}H_{36}O_{11}$, 584.2258).

4.3.10. (1S,2R,3R,4R,5S,7R,9S,10R)-2,3-Diacetoxy-9-benzoyloxy-1-(3-furoyloxy)-dihydro-β-agarofuran (**11**)

Colorless laquer; $[\alpha]_D^{25}$ +18.1 (*c* 0.3, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 230 (4.1) nm; IR ν_{max} (film) 2926, 2854, 1738, 1277, 1238, 1150, 1010, 757, 714 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.85 (3H, s, OAc-2), 2.26 (3H, s, OAc-3), OFu-1 [6.39 (1H, d, $J = 1.7$ Hz, H-4), 7.31 (1H, t, $J = 1.7$ Hz, H-5), 7.71 (1H, d, $J = 1.7$ Hz, H-2)], OBz-9 [7.45 (2H, m, H-3, H-5), 7.57 (1H, m, H-4), 8.07 (2H, d, $J = 7.7$ Hz, H-2, H-6)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.3 (d, C-4), 118.6 (s, C-3), 143.3 (d, C-5), 147.1 (d, C-2), 160.8 (s, COO−)], OBz-9 [127.7 (2 × d, C-3, C-5), 130.0 (2 × d, s, C-2, C-6, C-1), 132.5 (d, C-4), 165.4 (s, COO−)], OAc [(20.6 q, 170.4 s, OAc-2), (21.2 q, 171.0 s, OAc-3)], for other signals, see Table 2; EIMS *m/z* 568 $[M]^+$ (44), 553 (2), 446 (6), 354 (3), 311 (5), 232 (9), 157 (8), 105 (100), 95 (69); HREIMS *m/z* 568.2306 $[M]^+$ (calcd for $C_{31}H_{36}O_{10}$, 568.2308).

4.3.11. (1S,2R,4S,5R,7R,9S,10R)-2-Acetoxy-9-benzoyloxy-1-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**12**)

Colorless laquer; $[\alpha]_D^{25}$ +65.1 (*c* 0.6, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 231 (3.8) nm; IR ν_{max} (film) 3539, 2929, 2855, 1740, 1714, 1277, 1241, 1156, 1012, 757, 714 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.86 (3H, s, OAc-2), OFu-1 [6.35 (1H, s, H-4), 7.30 (1H, s, H-5), 7.67 (1H, s, H-2)], OBz-9 [7.46 (2H, m, H-3, H-5), 7.57 (1H, m, H-4), 8.04 (2H, d, $J = 7.5$ Hz, H-2, H-6)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.4 (d, C-4), 118.8 (s, C-3), 143.4 (d, C-5), 147.3 (d, C-2), 161.1 (s, COO−)], OBz-9 [127.9 (2 × d, C-3, C-5), 129.8 (s, C-1), 130.2 (2 × d, C-2, C-6), 132.9 (d, C-4), 165.6 (s, COO−)], OAc (20.9 q, 170.5 s, OAc-2), for other signals, see Table 2; EIMS *m/z* 526 $[M]^+$ (6), 510 (10), 465 (1), 388 (7), 362 (6), 329 (3), 232 (8), 105 (99), 95 (100); HREIMS *m/z* 526.2185 $[M]^+$ (calcd for $C_{29}H_{34}O_9$, 526.2203).

4.3.12. (1S,2R,4S,5R,7R,9S,10R)-2-Acetoxy-1,9-di-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**13**)

Colorless laquer; $[\alpha]_D^{25}$ +45.5 (*c* 0.2, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 274 (4.4), 223 (4.2) nm; IR ν_{max} (film) 3517, 2925, 2853, 1738, 1311, 1241, 1160, 1008, 874, 758 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.86 (3H, s, OAc-2), OFu-1 [6.45 (1H, s, H-4), 7.31 (1H, s, H-5), 7.76 (1H, s, H-2)], OFu-9 [6.70 (1H, s, H-4), 7.39 (1H, s, H-5), 8.02 (1H, s, H-2)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.3 (d, C-4), 118.7 (s, C-3), 143.0 (d, C-5), 147.1 (d, C-2), 161.7 (s, COO−)], OFu-9 [109.8 (d, C-4), 118.9 (s, C-3), 143.3 (d, C-5), 148.5 (d, C-2), 162.4 (s, COO−)], OAc (20.7 q, 170.5 s, OAc-2), for other signals, see Table 2; EIMS *m/z* 516 $[M]^+$ (14), 501 (10), 474 (1), 456 (2), 389 (3), 362 (5), 250 (4), 95 (100); HREIMS *m/z* 516.1978 $[M]^+$ (calcd for $C_{27}H_{32}O_{10}$, 516.1995).

4.3.13. (1S,2R,4S,5R,7R,9S,10R)-2-Acetoxy-9-trans-cynamoiloxy-1-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**14**)

Colorless laquer; $[\alpha]_D^{25}$ +124.7 (*c* 0.6, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 276 (4.1), 222 (4.0), 217 (4.0) nm; IR ν_{max} (film) 3536, 2931, 1740, 1310, 1241, 1166, 1009, 757, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.89 (3H, s, OAc-2), OFu-1 [6.49 (1H, d, $J = 1.6$ Hz, H-4), 7.30 (1H, t, $J = 1.6$ Hz, H-5), 7.80 (1H, d, $J = 1.6$ Hz, H-2)], OCin-9 [6.44 (1H, d, $J = 16.0$ Hz, H-α), 7.40 (3H, m, H-3, H-4, H-5), 7.52 (1H, d, $J = 16.0$ Hz, H-β), 7.56 (2H, m, H-2, H-6)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.6 (d, C-4), 119.1 (s, C-3), 143.6 (d, C-5), 147.4 (d, C-2), 161.6 (s, COO−)], OCin-9 [118.7 (d, C-α), 128.2 (2 × d, C-2, C-6), 128.8 (2 × d, C-3, C-5), 130.1 (d, C-4), 134.6 (s, C-1), 144.6 (d, C-β), 166.2 (s, COO−)], OAc (20.9 q, 170.4 s, OAc-2), for other signals, see Table 2; EIMS *m/z* 552 $[M]^+$ (19), 537 (5), 421 (2), 389 (23), 362 (6), 249 (7), 232 (6), 131 (100), 95 (67); HREIMS *m/z* 552.2380 $[M]^+$ (calcd for $C_{31}H_{36}O_9$, 552.2359).

4.3.14. (1S,2R,4R,5S,7R,9S,10R)-2-Acetoxy-9-benzoyloxy-1-(3-furoyloxy)-dihydro-β-agarofuran (**15**)

Colorless laquer; $[\alpha]_D^{25}$ +81.5 (*c* 0.2, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 230 (3.8) nm; IR ν_{max} (film) 2926, 2854, 1737, 1711, 1304, 1278, 1244, 1143, 1014, 757, 714 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.87 (3H, s, OAc-2), OFu-1 [6.37 (1H, s, H-4), 7.44 (1H, m, H-5), 7.70 (1H, s, H-2)], OBz-9 [7.45 (2H, m, H-3, H-5), 7.57 (1H, m, H-4), 8.06 (2H, d, $J = 7.7$ Hz, H-2, H-6)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.5 (d, C-4), 119.1 (s, C-3), 143.4 (d, C-5), 147.2 (d, C-2), 161.2 (s, COO−)], OBz-9 [127.9 (2 × d, C-3, C-5), 130.0 (s, C-1), 130.3 (2 × d, C-2, C-6), 132.8 (d, C-4), 165.8 (s, COO−)], OAc (21.1 q, 170.9 s, OAc-2), for other signals, see Table 2; EIMS *m/z* 510 $[M]^+$ (55), 495 (1), 450 (1), 388 (9), 346 (3), 277 (5), 234 (10), 105 (100), 95 (85); HREIMS *m/z* 510.2248 $[M]^+$ (calcd for $C_{29}H_{34}O_8$, 510.2254).

4.3.15. (1S,4S,5R,7R,9S,10S)-9-Benzoyloxy-1-(3-furoyloxy)-4-hydroxy-dihydro-β-agarofuran (**16**)

Colorless laquer; $[\alpha]_D^{25}$ +69.7 (*c* 0.6, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 230 (3.9) nm; IR ν_{max} (film) 3543, 2930, 1720, 1307, 1278, 1160, 1092, 970, 760, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ OFu-1 [6.34 (1H, s, H-4), 7.25 (1H, s, H-5), 7.63 (1H, s, H-2)], OBz-9 [7.38 (2H, m, H-3, H-5), 7.51 (1H, m, H-4), 7.91 (2H, d, $J = 7.7$ Hz, H-2, H-6)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ OFu-1 [109.4 (d, C-4), 119.2 (s, C-3), 143.2 (d, C-5), 147.1 (d, C-2), 162.1 (s, COO−)], OBz-9 [128.0 (2 × d, C-3, C-5), 129.7 (s, C-1), 129.8 (2 × d, C-2, C-6), 132.8 (d, C-4),

165.6 (s, COO–)], for other signals, see Table 2; EIMS *m/z* 468 $[M]^+$ (5), 453 (27), 356 (10), 346 (9), 234 (14), 105 (100), 95 (58); HREIMS *m/z* 468.2129 $[M]^+$ (calcd for $C_{27}H_{32}O_7$, 468.2148).

4.4. Biological assays

4.4.1. Chemicals

Vinblastine (VNB), verapamil, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium ortho-vanadate, and colchicine were purchased from Sigma–Aldrich (Madrid, Spain). Daunomycin (DNM) was from Pfizer (Madrid, Spain).

4.4.2. Cell cultures

The parental drug-sensitive NIH-3T3 cell line and the one transfected with human MDR1-G185 (MDR1 cells) were cultured in DMEM (Invitrogen, Barcelona, Spain) with 10% heat-inactivated fetal bovine serum (iFBS), with 2 mM L-glutamine, 250 U/mL penicillin G and 250 μg/mL streptomycin, as previously described [17].

4.4.3. Modulation of DNM efflux by sesquiterpenes

The sesquiterpenes studied in the present work were previously screened for their ability to block the Pgp-dependent DNM efflux by flow cytometry analysis as previously described [17]. Briefly, 24 h before the experiment, MDR1 cells in logarithmic phase of growth were seeded in 24-well plates at a density of 10^5 cells per well. Afterward, cells were incubated for 30 min at 37 °C in DMEM + 10% iFBS in the presence or absence of 10 μM sesquiterpenes with 2 μM DNM. Finally, cells were washed twice with ice-cold PBS, trypsinized and resuspended in 0.2 mL of ice-cold PBS for immediate analysis. Fluorescence measurements of individual cells were made with a Beckton Dickinson FacScan (BD European HQ, Belgium). To calculate K_i for inhibition of DNM efflux, cells were incubated with DNM as described above in the presence or absence of different concentrations of sesquiterpenes. All intracellular fluorescence values were converted to percentage inhibition of Pgp normalized for MDR1 cells treated with 5 mM ortho-vanadate (100% inhibition of Pgp), plotted and fitted to the Hill equation for allosteric interactions, using SigmaPlot 2000 software: $i = (I_{max} \times S^n)/(K_i + S^n)$; where *i* is the percentage inhibition of DNM efflux at a given sesquiterpene concentration, I_{max} is the maximal percentage inhibition, *S* is the concentration of sesquiterpene, and *n* is the Hill coefficient.

4.4.4. Reversion of Pgp-dependent DNM and VNB resistance

The dose–response curves of drug-sensitive and MDR1 cells were determined by the MTT colorimetric assay [18] as described previously [14]. To assess the chemosensitizing effect of the best sesquiterpenes blocking the DNM efflux activity of Pgp (compounds **1**, **2**, **4**, **6**, **8**, **9**, **11** and **14**), both drug-sensitive and MDR1 cells were exposed to increasing concentrations of DNM or VNB (up to 500 ng/mL) in the presence/absence of fixed concentrations (1, 3 and 10 μM) of each sesquiterpene. Dose–response curves were generated by non-linear regression (using SigmaPlot 2000 for Windows, SPSS Inc.) of the data points to a four-parameter logistic curve, in order to determine the IC_{50} (defined as the drug concentration that inhibit cell growth by 50%) for DNM and VNB at each sesquiterpene concentration. The reversal index for a given sesquiterpene at a given concentration is the ratio between the IC_{50} for MDR cells without sesquiterpene and the IC_{50} with sesquiterpene.

Acknowledgments

This study was supported by the Agencia Canaria de Investigación, Innovación y Sociedad de la Información (C200801000049), the Plan Andaluz de Investigación, Proyecto de Excelencia P08-CTS-03625 and by FEDER Funds from the EU (SC), and the Plan Andaluz de Investigación cod. BIO130 (FG). N.R.P. thanks the Gobierno Autónomo de Canarias for the fellowship. We also thank Dr. Ira Pastan (National Cancer Institute, NIH, Bethesda, MD) for providing the NIH-3T3 and NIH-3T3 MDR-G185 cell lines. We thank Dr J.T. Vázquez for carrying out the CD experiments. Thanks are also due to Alfredo Tupayachi for collecting the plant material.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2011.07.048.

References

[1] M. Ladanyi, C.W.P. Hogendoorn, J. Pathol. 223 (2011) 99–101.
[2] H.R. Mellor, R. Callaghan, Pharmacology 81 (2008) 275–300.
[3] Y. Li, H. Yuan, K. Yang, W. Xu, W. Tang, X. Li, Curr. Med. Chem. 17 (2010) 786–800.
[4] P.D.W. Eckford, F.J. Sharom, Chem. Rev. 109 (2009) 2989–3011.
[5] J. Molnár, H. Engi, J. Hohmann, P. Molnár, J. Deli, O. Wesolowska, K. Michalak, Q. Wang, Curr. Top. Med. Chem. 10 (2010) 1757–1768.
[6] C.P. Reyes, F. Muñoz-Martínez, I.R. Torrecillas, C.R. Mendoza, F. Gamarro, I.L. Bazzocchi, M.J. Núñez, L. Pardo, S. Castanys, M. Campillo, I.A. Jiménez, J. Med. Chem. 50 (2007) 4808–4817.
[7] D. Torres-Romero, F. Muñoz-Martínez, I.A. Jiménez, S. Castanys, F. Gamarro, I.L. Bazzocchi, Org. Biomol. Chem. 7 (2009) 5166–5172.
[8] F. Cortés-Selva, I.A. Jiménez, F. Muñoz-Martínez, M. Campillo, I.L. Bazzocchi, L. Pardo, A.G. Ravelo, S. Castanys, F. Gamarro, Curr. Pharm. Des. 11 (2005) 3125–3139.
[9] A.G. González, B.M. Tincusi, I.L. Bazzocchi, H. Tokuda, H. Nishino, T. Konoshima, I.A. Jiménez, A.G. Ravelo, Bioorg. Med. Chem. 8 (2000) 1773–1778.
[10] F. Muñoz-Martínez, C.R. Mendoza, I.L. Bazzocchi, S. Castanys, I.A. Jiménez, F. Gamarro, J. Med. Chem. 48 (2005) 4266–4275.
[11] N. Harada, K. Nakanishi, Circular Dichroism Spectroscopy Exciton Coupling in Organic Stereochemistry. University Science Books, Mill Valley, CA, 1983.
[12] PC Model from Serena Software, P.O. 3076, Bloomington, IN 47402-3076, version 9.2 with MMX force field.
[13] N.R. Perestelo, I.A. Jiménez, H. Tokuda, H. Hayashi, I.L. Bazzocchi, J. Nat. Prod. 73 (2010) 127–132.
[14] S.M. Kupchan, R.M. Smith, J. Org. Chem. 42 (1977) 115–118.
[15] C.R. Smith, R.W. Miller, D. Weisleder, W.K. Rohwedder, N. Eickman, J. Clardy, J. Org. Chem. 41 (1976) 3264–3269.
[16] A.G. González, M.P. Núñez, I.A. Jiménez, A.G. Ravelo, J.A. Sazatornil, J.T. Vázquez, I.L. Bazzocchi, E.Q. Morales, O.M. Muñoz, J. Chem. Soc., Perkin Trans. 1 (1992) 1437–1441.
[17] F. Muñoz-Martínez, P. Lu, F. Cortés-Selva, J.M. Pérez-Victoria, I.A. Jiménez, A.G. Ravelo, F.J. Sharom, F. Gamarro, S. Castanys, Cancer Res. 64 (2004) 7130–7138.
[18] T. Mosmann, J. Immunol. Methods 65 (1983) 55–63.